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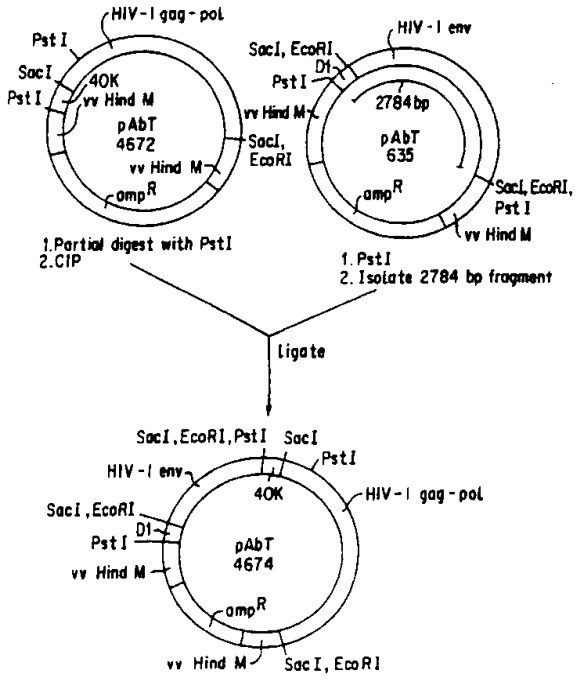
(57) Abstract

Recombinant viral vectors which coexpress heterologous polypeptides capable of assembling into defective nonself-propagating viral particles are disclosed. The viral vectors as well as the viral particles can be used as immunogens and for targeted delivery of heterologous gene products and drugs.



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SELF ASSEMBLED, DEFECTIVE, NONSELF-PROPAGATING VIRAL PARTICLES

Related Application

This application is a Continuation-in-part of U.S. Serial No. 07/540,109, filed June 19, 1990, which is a Continuation-in-part of U.S. Serial No. 07/360,027, filed June 1, 1989, which is a Continuation-in-part of U.S. Serial No. 07/205,454, filed June 10, 1988. The teachings of each application are incorporated herein by reference.

Background

Recombinant approaches have been used in attempts to develop vaccines against diseases for which no vaccine currently exists, or for which conventional vaccine approaches are less desirable. For example, since the human immunodeficiency virus (HIV) was first identified as the etiologic agent of Acquired Immunodeficiency Disease Syndrome (AIDS), (Barre-Sinoussi, et al., Science 220:868 (1983); Levey, et al., Science 225:840 (1984); Gallo, et al., Science 224:500 (1984)), considerable effort has been directed towards the development of a safe and effective vaccine.

The human immunodeficiency viruses, HIV-1 and HIV-2, are members of the lentivirus subclass of retroviruses. Gonda, et al., Science 227:173 (1985); Sonigo, et al., Cell 42:369 (1985). The virus particles contain an inner core comprised of capsid proteins (encoded by the viral gag gene) that encase the viral RNA genome. Rabson & Martin, Cell 40:477 (1985). The central core is surrounded by a lipid envelope that contains the viral-encoded envelope glycoproteins. Virus-encoded enzymes required for replication, such as the reverse transcriptase and integrase (encoded by the pol gene), are also incorporated into the virus particle.

There are obvious difficulties with the use of whole virus for an HIV vaccine. The fear that an attenuated virus could revert to virulence, and the danger of incomplete inactivation of killed virus preparations, together with the reluctance to introduce the HIV genome into seronegative individuals have argued against the uses of live attenuated or killed HIV vaccines for the prevention of infection.

Advances in recombinant DNA technology may make it possible to use heterologous expression systems for the synthesis not only of individual antigens, but also of defective, nonself-propagating, virus-like particles. It has been demonstrated that capsid proteins of certain viruses can assemble into particles morphologically and immunologically similar to the corresponding virus. For example, the P1 precursor of several picornaviruses synthesized in vitro can be processed into individual capsid proteins which then assemble into immunoreactive virion-like particles. Nicklin, et al., Biotechnology 4:33 (1986); Palmenberg, et al., J. Virol. 32:770 (1979); Shih, et al., Proc. Natl. Acad. Sci. USA 75:5807 (1978); Hanecak, et al., Proc. Natal. Acad. Sci. USA 79:3973 (1982); Grubman, et al., J. Virol. 56:120 (1985). Self-assembly of capsid proteins expressed in vivo in several recombinant expression systems has also been reported. For example, when human hepatitis B surface antigen is expressed in yeast cells, the polypeptide assembles into particles similar in appearance to those isolated from human plasma (Valenzuela, et al., Nature 298:347 (1982)); these particles stimulate anti-hepatitis B antibody production in several species and can protect chimpanzees from virus challenge. McAleer, et al., Nature 307:178 (1984).

In another example, it was shown that coexpression of canine parvovirus (CPV) capsid proteins VP1 and VP2 in murine cells transformed with a bovine papilloma virus/CPV recombinant plasmid resulted in the formation of self-assembling virus-like particles (Mazzara, et al., 1986 in Modern Approaches to

Vaccines, Cold Spring Harbor Laboratory, N.Y.; R.M. Chanock and R.A. Lerner, eds. pp. 419-424; Mazzara, et al., U.S. Patent Application No. 905,229, filed September 8, 1986); when used to vaccinate susceptible dogs, these empty capsids elicited immune responses capable of protecting against CPV challenge. It has also been shown that the HIV-1 and SIV p55gag precursor polypeptides expressed in Spodoptera frugiperda cells using a baculovirus expression vector assembles into virus-like particles which are secreted into the cell culture medium. Gheysen, et al., Cell 59:103 (1989); Delchambre, et al., The EMBO J. 8:2653-2660 (1989).

Summary of the Invention

This invention pertains to recombinant viral vectors capable of expressing at least two different polypeptides of a heterologous virus capable of self-assembly, in vivo or in vitro, into defective, non-self propagating viral particles, and to methods of producing the recombinant virus. Preferably, the viral particles are produced by vaccinia viral vectors that coexpress the env and gag-pol genes of HIV. This invention also pertains to intermediate DNA vectors which recombine with a parent virus in vivo or in vitro to produce the recombinant viral vector, and to methods of vaccinating a host with the recombinant viral vector to elicit protective immunity against the correlate heterologous pathogenic virus. In addition, this invention pertains to defective, nonself-propagating viral particles, such as lentivirus or picornavirus particles, produced by the recombinant viral vectors. These viral particles may be isolated and used themselves as immunogens or in combination with other immunogens for vaccination against pathogenic viruses, or for therapeutic purposes, such as enhancing immune responses in an infected individual, or for targeted delivery of heterologous nucleic acids and/or therapeutic agents, such as cytotoxic drugs, to specific cell

types. The viral particles can have substantially little or no RNA packaged within the particle; or they can contain specific RNA for delivery of heterologous genes to a targeted cell.

Brief Description of the Figure

Figure 1a shows the construction of plasmid pAbT4672 containing the HIV-1 gag-pol gene under the control of the vaccinia 40K promoter.

Figure 1b shows the construction of plasmid pAbT635 containing the HIV-1 env gene under the control of the vaccinia D1 promoter.

Figure 1c shows the construction of pAbT4674, a plasmid vector for the insertion and expression of HIV gag-pol (strain HXB2) and env (strain BH10) in vaccinia virus. pAbT4674 contains the gag-pol gene under the control of the vaccinia 40K promoter and the env gene under the control of the vaccinia D1 promoter.

Figures 2a and 2b show the construction of plasmid pAbT4716, a plasmid vector for in vivo recombination with vaccinia virus. pAbT4716 contains the HIV env gene under the transcriptional direction of the vaccinia 40K promoter.

Figure 3 shows the sequence changes resulting from the mutagenesis of plasmid pAbT8068.

Figures 4a and 4b show the construction of plasmid pAbt4704, a plasmid vector for in vivo recombination with vaccinia virus. pAbT4704 contains the HIV gag-pol genes under the transcriptional direction of the vaccinia 40K promoter.

Detailed Description of the Invention

1. Genes encoding viral antigens

Genes encoding viral polypeptides capable of self assembly

into defective, nonself-propagating viral particles can be obtained from the genomic DNA of a DNA virus or the genomic cDNA of an RNA virus or from available subgenomic clones containing the genes. These genes will include those encoding viral capsid proteins (i.e., proteins that comprise the viral protein shell) and, in the case of enveloped viruses, such as retroviruses, the genes encoding viral envelope glycoproteins. Additional viral genes may also be required for capsid protein maturation and particle self-assembly. These may encode viral proteases responsible for processing of capsid protein or envelope glycoproteins.

As an example, the genomic structure of picornaviruses has been well characterized, and the patterns of protein synthesis leading to virion assembly are clear. Rueckert, R. in Virology (1985), B.N. Fields, et al., (eds.) Raven Press, New York, pp 705-738. In picornaviruses, the viral capsid proteins are encoded by an RNA genome containing a single long reading frame, and are synthesized as part of a polyprotein which is processed to yield the mature capsid proteins by a combination of cellular and viral proteases. Thus, the picornavirus genes required for capsid self-assembly include both the capsid structural genes and the viral proteases required for their maturation.

Another virus class from which genes encoding self-assembling capsid proteins can be isolated is the lentiviruses, of which HIV is an example. Like the picornaviral capsid proteins, the HIV gag protein is synthesized as a precursor polypeptide that is subsequently processed, by a viral protease, into the mature capsid polypeptides. However, the gag precursor polypeptide can self-assemble into virus-like particles in the absence of protein processing. Gheysen, et al., Cell 59:103 (1989); Delchambre, et al., The EMBO J. 8:2653-2660 (1989). Unlike picornavirus capsids, HIV capsids are surrounded by a loose membranous envelope that contains the viral glycoproteins. These are encoded by the viral env gene.

The examples illustrate the use of HIV genes selected for

expression in recombinant viruses of this invention. These genes and their protein products are outlined in Table 1. The three major virion components derived from the env, gag, and pol genes are synthesized as precursor polyproteins which are subsequently cleaved to yield mature polypeptides as outlined below in Table 1.

Table 1

HIV Genes for Recombinant into Pox Virus

Gene	Gene Product	Processed Peptides
env	gp160	gp120 extracellular membrane protein
		gp41 transmembrane protein
gag	p55	p24
		p17 capsid proteins
		p15
pol	p160*	p10 protease
		p66/p51 reverse transcriptase
		p31 endonuclease

* Part of the gag-pol product.

2. Parent Viruses

A number of viruses, including retroviruses, adenoviruses, herpesviruses, and pox viruses, have been developed as live viral vectors for the expression of heterologous antigens. Cepko, et al., Cell 37:1053-1062 (1984); Morin, et al., Proc. Natl. Acad. Sci. USA 84:4626-4630 (1987); Lowe, et al. Proc. Natl. Acad. Sci. USA 84:3896-3900 (1987); Panicali & Paoletti, Proc. Natl. Acad. Sci. USA 79:4927-4931 (1982); Mackett, et al., Proc. Natl. Acad. Sci. USA 79:7415-7419 (1982). The examples given illustrate the use of the pox virus family. The preferred pox virus is vaccinia virus, a relatively benign virus which has been used for years as a vaccine against smallpox. Vaccinia virus has been developed as an infectious eukaryotic cloning vector (Paoletti and Panicali, United States Patent No. 4,603,112) and recombinant vaccinia virus has been used successfully as a vaccine in several experimental systems. The virus is considered non-oncogenic, has a well-characterized genome, and can carry large amounts of foreign DNA without loss of infectivity. Mackett, M. and G.L. Smith, J. Gen. Virol. 67:2067 (1986). Another preferred pox virus is fowl pox virus, a pathogen of poultry. This virus has also been developed into a eukaryotic cloning vector. Boyle, et al., Gene 35:169-177 (1985); U.S. Patent Application Serial No. 07/398,762, filed August 25, 1989.

3. DNA vectors for in vivo recombination with a parent virus

According to the method of this invention, viral genes that code for polypeptides capable of assembly into viral particles are inserted into the genome of a parent virus in such as manner as to allow them to be expressed by that virus along with the expression of the normal complement of parent virus proteins. This can be accomplished by first constructing a DNA donor vector for in vivo recombination with a parent virus.

In general, the DNA donor vector contains the following elements:

a) a prokaryotic origin of replication, so that the vector may be amplified in a prokaryotic host;

b) a gene encoding a marker which allows selection of prokaryotic host cells that contain the vector (e.g., a gene encoding antibiotic resistance);

c) at least two heterologous viral genes (e.g., HIV or picornavirus genes), each gene located adjacent to a transcriptional promoter (e.g., the vaccinia 7.5K, 30K, 40K, 11K or BamF promoters or modified versions of these promoters) capable of directing the expression of adjacent genes; and

d) DNA sequences homologous to the region of the parent virus genome where the foreign gene(s) will be inserted, flanking the construct of element c (e.g., the vaccinia TK or HindIII M sequences).

Methods for constructing donor plasmids for the introduction of multiple foreign genes into pox virus are described in U.S. Patent Application Serial No. 910,501, filed September 23, 1986, entitled "Pseudo-rabies Vaccine", the techniques of which are incorporated herein by reference. In general, all viral DNA fragments for construction of the donor vector, including fragments containing transcriptional promoters and fragments containing sequences homologous to the region of the parent virus genome into which foreign genes are to be inserted, can be obtained from genomic DNA or cloned DNA fragments.

The donor vector preferably contains an additional gene which encodes a selectable marker under control of a separate promoter which will allow identification of recombinant viruses containing inserted foreign DNA. Several types of marker genes can be used to permit the identification and isolation of recombinant viruses. These include genes that encode antibiotic

or chemical resistance (e.g., see Spyropoulos, et al., J. Virol. 62:1046 (1988); Falkner and Moss, J. Virol. 62:1849 (1988); Franke, et al., Mol. Cell. Biol. 5:1918 (1985)), as well as genes, such as the E. coli lacZ gene, that permit identification of recombinant viral plaques by colorimetric assay. Panicali, et al., Gene 47:193-199 (1986).

A method for the selection of recombinant vaccinia viruses relies upon a single vaccinia-encoded function, namely the 29K host-range gene product. Gillard, et al., Proc. Natl. Acad. Sci. USA 83:5573 (1986). This method was described in U.S. Patent Application Serial No. 205,189, filed June 20, 1988, entitled "Methods of Selecting for Recombinant Pox Viruses", the teachings of which are incorporated herein by reference.

4. Integration of foreign DNA sequences into the viral genome and isolation of recombinants

Homologous recombination between donor plasmid DNA and viral DNA in an infected cell results in the formation of recombinant viruses that incorporate the desired elements. Appropriate host cells for in vivo recombination are generally eukaryotic cells that can be infected by the virus and transfected by the plasmid vector. Examples of such cells suitable for use with a pox virus are chick embryo fibroblasts, HuTK143 (human) cells, and CV-1 and BSC-40 (both monkey kidney) cells. Infection of cells with pox virus and transfection of these cells with plasmid vectors is accomplished by techniques standard in the art (Panicali and Paoletti, United States Patent No. 4,603,112).

Following in vivo recombination, recombinant viral progeny can be identified by one of several techniques. For example, if the DNA donor vector is designed to insert foreign genes into the parent virus thymidine kinase (TK) gene, viruses containing integrated DNA will be TK^- and can be selected on this basis (Mackett, et al., Proc. Natl. Acad. Sci. USA 79:7415 (1982)).

Alternatively, co-integration of a gene encoding a marker or indicator gene with the foreign gene(s) of interest, as described above, can be used to identify recombinant progeny. One preferred indicator gene is the E. coli lacZ gene: recombinant viruses expressing beta-galactosidase can be selected using a chromogenic substrate for the enzyme (Panicali, et al., Gene 47:193 (1986)). A second preferred indicator gene for use with recombinant vaccinia virus is the vaccinia 29K gene: recombinant viruses that express the wild type 29K gene-encoded function can be selected by growth on RK13 cells. Another method by which recombinant viruses containing genes of interest can be identified is by an in situ enzyme based immunoassay in which protein expressed by vaccinia-infected cells is detected by the formation of live black plaques.

As described more fully in the Examples, donor plasmids containing HIV genes were recombined into vaccinia at the HindIII M region and recombinant viruses were selected as described above.

5. Characterizing the viral antigens expressed by recombinant viruses

Once a recombinant virus has been identified, a variety of methods can be used to assay the expression of the polypeptide encoded by the inserted gene. These methods include black plaque assay (an in situ enzyme immunoassay performed on viral plaques), Western blot analysis, radioimmunoprecipitation (RIPA), and enzyme immunoassay (EIA). Antibodies to antigens expressed by viral pathogens are either readily available, or may be made according to methods known in the art. For example, for human immunodeficiency virus, the antibodies can be either sera from human patients infected with HIV, or commercially available monoclonal antibodies directed against specific HIV polypeptides.

6. Viral Particle formation

Expression analysis described in the preceding section can be used to confirm the synthesis of the polypeptides encoded by inserted heterologous viral genes, but does not address the question of whether these polypeptides self-assemble, in vivo or in vitro, into defective viral particles. Two experimental approaches can be used to examine this issue.

The first approach is to visually examine by electron microscopy lysates of cells infected with recombinant viruses that express one or more viral polypeptides. The presence of retroviral envelope glycoproteins on the surface of the particles can be demonstrated with immunogold electron microscopy, using a monoclonal antibody directed against one of the envelope glycoproteins.

In order to characterize the defective viral particles produced by recombinant viruses expressing viral polypeptides, these particles can be isolated by high speed centrifugation from the culture medium of cells infected with the recombinant viruses in the presence of [^{35}S]-methionine. The pellet resulting from centrifugation of the culture medium can be resuspended and both the pellet and the supernatant can be immunoprecipitated with an appropriate antiserum to analyze the viral polypeptides present in each fraction. For example, in the case of recombinants expressing HIV polypeptides, human anti-HIV antisera (for vaccinia/HIV recombinants) can be used for the analysis.

To further characterize the material in the pellet resulting from centrifugation of the culture medium, the pellet can be resuspended and analyzed on a sucrose gradient. The gradient can then be fractionated and the fractions immunoprecipitated with the appropriate antiserum. These experiments show whether the pellet contains material banding at the density expected for defective viral particles.

These methods can also be used to determine whether

expression of viral polypeptides directed by two different viruses present in the same infected cell gives rise to the production of defective viral particles. For example, these experiments can be performed using cells coinfected in vitro with one recombinant expressing gag and a second recombinant expressing env. The simultaneous expression in a single cell of both env and gag polypeptides, whether directed by a single divalent recombinant virus or by two different monovalent viruses, would be expected to result in the formation of defective retroviral particles that contain a protein core comprising gag polypeptides surrounded by an envelope containing virally-encoded envelope glycoproteins.

7. Production of virus-like particles which do not contain RNA

Two approaches can be employed to produce "empty" virus-like particles having substantially little or no RNA packaged within the capsid. Such particles may provide greater potential safety for use as a subunit vaccine because the level of viral RNA is reduced, particularly in the case of HIV-like particles.

The first approach involves the removal of gag-specific sequences responsible for recognition of RNA. Using standard molecular biology techniques, it is possible to generate DNA virus recombinants, such as pox virus, that contain and express HIV-1 gag genes having point or deletion mutations in the nucleocapsid domain of the gag protein. For example, pox virus-expressed mutant gag gene products produced by this method will retain the ability to assemble into virus-like particles, but will substantially lack the ability to package mRNA containing the packaging sequence. This approach is based upon previous findings that all retroviruses contain, the nucleocapsid domain of the gag protein, at least one copy of the sequence Cys-X_2-Cys-X_4-His-X_4-Cys which is involved in the

recognition of genomic RNA. Point and deletion mutations generated in this motif in HIV-1 (Gorelick, et al., J. Cell Biochem., Suppl. 14D:150 (1990)) and Moloney MuLV (Gorelick, et al., Proc. Natl. Acad. Sci. USA 85:8420 (1988)) demonstrate that the mutant viruses produce viral particles deficient in genomic RNA. Thus, similar point or deletion mutations can be used to generate pox virus recombinants capable of producing empty viral-like particles of this invention.

The second approach is to remove the cis-acting packaging sequences from the gag-pol mRNA. These cis-acting sequences which are located between the 5'LTR and the gag gene initiation codon in avian and murine retroviruses are essential for efficient packaging of viral RNA into virions. A sequence required for effective packaging of the HIV-1 genome RNA in viral particles was recently identified. Lever, et al., J. Virol. 63:4085 (1989). Lever, et al., demonstrated that deletion of 19 base pairs between the 5' LTR and the gag gene initiation codon of HIV-1 resulted in the generation of a mutant virus markedly attenuated for replication in human T lymphocytes. The mutant virus was characterized by nearly wild-type ability to encode viral proteins and to produce virion particles. These virion particles exhibited a significant reduction in the content of HIV-1 specific RNA.

The vaccinia recombinants shown in the Examples (vAbT408) encode a gag-pol mRNA that contains the putative packaging sequence identified by Lever, et al. However, it is possible to generate pox virus recombinants that contain and express the gag-pol coding sequence but which do not contain, as part of the inserted gene, the cis-acting packaging sequences 5' to the start of the gag initiation codon. Such recombinant pox virus should retain the ability to produce HIV-1 virus-like particles, since it will express the entire gag structural gene sequence. These particles, however, should contain significantly lower levels of HIV-1 specific RNA as compared to particles produced by recombinants which expresses an mRNA that contains this

cis-acting packaging sequence, such as recombinant vAbT408 shown in the Examples.

8. Production of virus-like particles that contain a specific expressible RNA that can be reverse transcribed

Alternatively, it may be preferable to produce a capsid that contains a specific RNA packaged therein. Such capsids can be used to deliver specific RNA to cells targeted by the particles (i.e., HIV-infectible cells). Preferably, this can be done by minimizing the level of gag-pol messenger RNA packaged in the capsids and at the same time encoding the desired RNA species, in a recombinant DNA virus (the same one in a multi-valent formulation, or a different one for use in coinfection), such as pox virus.

Using standard techniques, it is possible to design a recombinant that produces particles 1) containing an RNA of choice; 2) capable of targeting HIV-1 infectible cells; 3) capable of delivering the RNA to those cells; 4) capable of reverse transcribing that RNA into DNA which can integrate into the genome of the targeted cell. The packaged RNA species should contain the HIV LTRs, so that it can be reverse-transcribed by the reverse transcriptase contained in the particle into DNA, and that DNA should be capable of integrating into the host genome. The RNA of choice should also contain the cis-acting packaging sequence and a gene of interest under the transcriptional control of either the HIV-1 LTRs or of a heterologous eukaryotic promoter (e.g., the CMV IE or the SV40 promoter).

One way to produce such an RNA in pox virus-infected cells is to insert the appropriate sequences into pox virus under the transcriptional control of a pox virus promoter (alternatively, the sequences, under the transcriptional control of a pox promoter, can be carried on a plasmid that is transfected into pox virus-infected cells). The construct to be expressed in pox

virus-infected cells should have the following elements: 1) sequence capable of encoding an RNA containing (in the 5' to 3' orientation) the leftward HIV-1 LTR, the cis-acting packaging sequences, the gene of interest (or, a eukaryotic promoter linked to the gene of interest) and the rightward HIV-1 LTR; 2) these sequences should be under the transcriptional control of a pox virus early promoter and linked to this promoter in such a way that the RNA cap site specified by the pox virus promoter coincides with the authentic RNA cap site of HIV-1; 3) these sequences should be flanked at the 3' terminus by a pox virus RNA termination sequence (TTTTTNT), if it is desirable, so that an RNA of a discrete length is encoded. For insertion into recombinant pox virus, elements 1-3 should be flanked by sequences homologous to a non-essential region of the pox virus genome, to permit insertion of these sequences into the genome by homologous recombination in vivo. Production of this RNA in cells infected by a recombinant pox virus capable of directing the formationof HIV-1 virus-like particles which containenv, gag and pol gene products will result in the production of particles that contain the inserted RNA of interest. It may be possible to maximize the proportion of particles containing the desired RNA by using a pox virus recombinant which does not package substantial amounts of mRNA encoding the gag-pol genes by using certain drugs, such as araC, which permit RNA synthesis only from early vaccinia promoters; and/or by using recombinant vaccinia viruses which contain conditionally lethal mutations that prevent the synthesis of late RNA.

9. Production of virus-like particles that contain a specific expressible RNA that cannot be reverse transcribed

It may be desirable to package RNA that can, when delivered to the target cell, be translated directly by the cell rather than integrated into the cellular DNA. For example, RNA encoding a highly active toxin might be delivered in a

virus-like particle as translatable mRNA. To produce particles containing non-integrating mRNA, the recombinant can be designed essentially as described in 8, above, except that neither the HIV-1 LTRs nor the heterologous eukaryotic promoter are included in the construction. All other elements of the construction are identical. Without the LTRs, the RNA cannot be reverse transcribed and will be available to the cell for translation into protein.

10. Vaccines

Live recombinant viral vectors that express heterologous viral antigens capable of self-assembly into defective non-self-propagating virus particles can be used to vaccinate humans or animals susceptible to infection if the viral vector used to express the heterologous defective virus particles infects but does not cause significant disease in the vaccinated host. Examples of such benign viral vectors include certain pox viruses, adenoviruses, and herpes viruses. For example, vaccination with live recombinant vaccinia virus is followed by replication of the virus within the host. During replication, the viral genes are expressed along with the normal complement of recombinant virus genes. Thus, during the two-week post-immunization period when the live recombinant virus is replicating (Fenner, F., in Virology, Fields, et al., eds. Raven Press, New York, 1985, pp 661-684), viral antigens may be presented to the host immune system in a manner that closely mimics the presentation of antigens in an authentic viral infection, that is, as defective, non-self-propagating viral particles extremely similar to the native virus. Viral antigens repeatedly presented both as free particles and in association with recombinant virus-infected cells may have the potential to prime the immune system to recognize and eliminate the virus during the early events of viral infection.

Alternatively, the defective virus particles produced by

these recombinant vector viruses can be isolated from cells infected in vitro with the recombinant vector viruses and from the culture medium of these infected cells, and themselves used for vaccination of individuals susceptible to viral infection. These particles resemble the native virus, but will not contain infectious viral genetic material, such as HIV mRNA. Consequently, they offer the advantage of conventional killed virus vaccine preparations: the ability authentically to present immunogenic antigens to the immune system of the vaccinated host. At the same time such particles circumvent the major drawbacks to the use of killed virus as a vaccine for the prevention of infection, including the danger of incomplete inactivation of killed virus preparations and, as for the case of certain viruses, such as retroviruses, the reluctance to introduce a complete viral genome (the HIV genome, for example) into seronegative individuals.

Vaccine compositions utilizing these defective virus particles would generally comprise an immunizing amount of the viral particles in a pharmaceutically acceptable vehicle. The vaccines would be administered in a manner compatible with the dosage formulation, and in such amount as would be therapeutically effective and immunogenic.

Finally, the purified particles may be used in combination with live recombinant viruses as part of a total vaccination protocol, either as the primary immunizing agent, to be followed by vaccination with live recombinant virus, or to boost the total immune response after primary vaccination with live recombinant virus.

11. Therapeutic use of recombinant viruses expressing viral antigens capable of assembling into defective viral particles; therapeutic use of defective viral particles produced by these recombinant viruses.

Even if immunization can not protect against infection,

immunization of a previously infected individual might prolong the latency period of that virus within the individual. This may be particularly important in the case of viral infections characterized by long latency periods, such as HIV infection. The long incubation time between HIV infection and the development of clinical AIDS may be due to an immune response to the initial infection which persists with health and wanes with disease. If this is the case, boosting the immune response by immunization with HIV antigen/parent virus recombinants that produce retroviral-like particles, or alternatively, with the purified particles themselves, may prevent the development of disease and reduce contagiousness. Salk, Nature 327:473 (1987).

These defective viral particles of this invention can also be used to deliver heterologous genes (e.g., antisense genes, mutant HIV genes) to a targeted cell. Specific RNA packaged within the defective viral particle can then be transcribed by the targeted cells. For example, the viral particle can contain a specific RNA that when transcribed is toxic to the targeted cell, which might be for example HIV-infected cells.

Viral particles containing RNA encoding heterologous genes can be administered to an individual to produce an immune response to the viral particles, as well as to deliver the heterologous gene products.

12. Therapeutic use of defective virus particles as agents for targeted drug delivery

Defective, nonself-propagating virus particles can also be used to deliver certain drugs (e.g., cytotoxic drugs, antiviral agents, nucleic acids) to virus receptor-bearing cells. Such drugs may be coupled, by techniques known in the art, to the outer surface of the virus particle, or incorporated within, and delivered with high specificity to target cells. For example, cytotoxic drugs may be coupled to defective HIV particles and delivered with a high degree of specificity to $CD4^+$ T cells,

since the HIV envelope glycoprotein present on these particles bind specifically and with high affinity to the CD4 molecule. Similarly, poliovirus particles, for example, preferentially bind cells of the nasopharynx and gut, and thus can be used to direct delivery of specific agents to these or other cells that have poliovirus receptors.

13. Diagnostic uses of virus-like particles

Immunogenic virus-like particles can be used to diagnose viral infection. The particles can be used to raise a panel of monoclonal antibodies and polyclonal antisera which recognize various epitopes on the virion. These monoclonal and/or polyclonal antibodies can be used individually or together as capture antibodies for an immunoassay to detect the presence of virus in urine, blood, or other physiological samples.

Alternatively, the particles themselves can be used as antigens for an immunoassay to detect the presence of antibody in urine, blood, or other physiological samples. Particularly preferred immunoassays are solid phase immunometric assays (enzymetric, radiometric). In such assays, the virus-like particle is immobilized on a solid phase to provide an immunoadsorbent. The techniques for use of solid phase immunoadsorbents are known in the art.

This invention is illustrated further by the following examples:

EXAMPLES

GENERAL PROCEDURES

Cells and Virus

E. coli strain MC1061 (Casadaban and Cohen, J. Mol. Biol. 138:179 (1980)) was used as the host for the growth of all plasmids. The monkey kidney cell line BSC-40 (Brockman &

Nathans, Proc. Natl. Acad. Sci. USA 71:942 (1974)) and the rabbit kidney cell line RK13 (ATCC No. CCL37; Beale, et al., Lancet 2:640 (1963)) were used for vaccinia infections and transfections. Cells were propagated in Dulbecco modified Eagles Medium (DME, Hazelton, Lexena, KS) supplemented with 5% fetal calf serum (FSC. Hyclone Laboratories, Inc., Logan, VT).

A plaque purified isolate of the Wyeth strain of vaccinia virus was obtained from Flow Laboratories (McLean, VA). This virus and 29K- lacZ+ strain vAbT33 (see U.S. Patent Application Serial No. 205,189, filed June 10, 1988, incorporated herein by reference) were used as the parental virus for in vivo recombination. Viral infection, transfections, plaque purification and virus amplification were performed essentially as described. Spyropoulos, et al., J. Virol. 62:1046 (1988).

Molecular Cloning Procedures

Restriction enzyme digestions, purification of DNA fragments and plasmids, treatment of DNA with Klenow, T4 DNA polymerase, calf intestinal alkaline phosphatase, T4 DNA ligase, or linkers and transformation of E. coli were performed essentially as described (Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982, the teachings of which are incorporated herein by reference). Restriction enzymes were obtained from New England Biolabs or Boehringer-Mannheim. The large fragment of DNA polymerase (Klenow) was obtained from United States Biochemical Corporation, T4 DNA polymerase was obtained from New England Biolabs, and T4 DNA ligase and calf intestinal alkaline phosphatase were obtained from Boehringer-Mannheim.

EXAMPLE 1

Construction of recombinant plasmid for in vivo recombination with vaccinia virus containing the HIV env gene under the

control of the vaccinia D1 promoter and the HIV gag-pol genes under the control of the vaccinia 40K promoter.

pHXBc2 is a plasmid that contains portions of the HIV-1 strain HXB2 genome; it was obtained from Dr. Joseph Sodroski of the Harvard Medical School. The construction and structure of plasmid pAbT4587 is described in U.S. Patent Application Serial No. 229,343, filed August 5, 1988. The construction and structure of plasmid pAbT4603 was described in U.S. Patent Application Serial No. 360,027, filed June 1, 1989. The teachings of these applications are incorporated herein by reference.

pAbT4674 (Figure 1).

a. Construction of pAbT4672 (Figure 1a).

Plasmid pAbT4587 was digested with SacI and treated with calf intestinal alkaline phosphatase (CIP). This vector was ligated to a 5354 bp fragment produced by digesting pHXBc2 with SacI, to yield plasmid pAbT4672.

b. Construction of pAbT635 (Figure 1b).

Plasmid vector pAbT4587 contains the vaccinia 40K promoter. Rosel, J. Virol. 60:436 (1986). Plasmid pAbT628 is identical to plasmid pAbT4587, except that it contains, in place of the 40K promoter, a DNA fragment with the following nucleotide sequence between the unique PstI and BamHI sites in the vector.

PstI

CTGCAGCAGC TTAAAATAGC TCTAGCTAAA GGCATAGATT ACGAATATAT

AAAAGACGCT TGTTAATAAG TAAATGAAAA AAAACTAGTC GTTTATAATA

BamHI

AAACACGATA TCTAGAGGAT CC

This DNA fragment contains vaccinia DNA sequences corresponding

to the D1 promoter (Niels, et al., Virology 153:96 (1986)), indicated by the underline, modified by the addition of linkers at each end to facilitate cloning into the plasmid vector. pAbT628 was digested with SacI, then treated with CIP. This vector was ligated to an approximately 2680 bp fragment containing this HIV env gene from HIV-1 strain BH10, which was produced by digestion of pAbT4603 with SacI, to yield plasmid pAbT629. Plasmid pAbT629 was partially digested with EcoRI, then ligated to an oligonucleotide linker containing a PstI site (New England Biolabs, Beverly, MA, cat. #1013). The resulting plasmid was designated pAbT635.

c. Construction of pAbT4674 (Figure 1c).

Plasmid pAbT635 was digested with PstI, and a 2784 bp fragment resulting from this digestion was purified. This fragment was ligated to the products of limited digestion of pAbT4672 with PstI, to produce the plasmid pAbT4674, which is a vector for the insertion and expression of HIV gag-pol (strain HXB2) and env (strain BH10) in vaccinia virus. pAbT4674 contains the gag-pol gene under the control of the vaccinia 40K promoter (Rosel, J. Virol. 60:436 (1986)), and the env gene under the control of the vaccinia D1 promoter. Niels, et al., Virology 153:96 (1986). The HIV genes and their adjacent vaccinia promoters are flanked by vaccinia DNA for directing recombination into the vaccinia HindIII M region. The vector DNA includes the 29K host-range gene for selection of vaccinia recombinants and a bacterial replicon and ampicillin-resistance gene for growth and selection in E. coli.

EXAMPLE 2

Construction of recombinant vaccinia viruses containing the HIV-1 (strain BH10) env gene under the control of the vaccinia D1 promoter and the HIV-1 (strain HXB2) gag-pol genes under the control of the vaccinia 40K promoter.

In vivo recombination (IVR) is a method whereby recombinant vaccinia viruses are created. Nankano, et al., Proc. Natl. Acad. Sci. USA 79:1593 (1982); Paoletti and Panicali, U.S. Patent No. 4,603,112. These recombinant viruses are formed by transfecting DNA containing a gene of interest into cells which have been infected by vaccinia virus. A small percent of the progeny virus will contain the gene of interest integrated into a specific site on the vaccinia genome. These recombinant viruses can express genes of foreign origin. Panicali and Paoletti, Proc. Natl. Acad. Sci. USA 79:4927 (1982); Panicali, et al., Proc. Natl. Acad. Sci. USA 80:5364 (1983).

a. Insertion of HIV-1 genes into vaccinia strain vAbT33.

To insert HIV-1 genes into the vaccinia virus genome at the HindIII M region of vaccinia virus strain vAbT33, a selection scheme based upon the 29K host-range gene, which is located in this region, was used. Gillard, et al., Proc. Natl. Acad. Sci. USA 83:5573 (1986). Recombinant vaccinia virus vAbT33 contains the lacZ gene in place of a portion of the 29K gene. This lacZ insertion destroys the function of the 29K gene; therefore, vAbT33 grows poorly on RK-13 cells, which require the 29K gene product. Furthermore, vAbT33 forms blue plaque on permissive cells in the presence of the chromogenic substrate for β-galactosidase, Bluogal, due to the presence of the lacZ gene. See U.S. Patent Application Serial No. 205,189, filed June 10, 1988.

IVR vector pAbT4674 was transfected into BSC-40 cells which had been infected with vaccinia virus vAbT33. Viral infection and plasmid transfection were performed essentially as described. Spyropoulos, et al., J. Virol. 62:1046 (1988). Recombinant viruses were selected as white plaques in the presence of Bluogal on RK-13 cells. Plaques were picked and purified, and the final recombinant, designated vAbT408, was amplified on RK-13 cells and purified over a 36% sucrose

cushion.

b. Insertion of HIV-1 genes into the Wyeth strain of vaccinia virus.

In order to insert HIV-1 genes in the vaccinia virus genome at the HindIII M region of the Wyeth vaccinia virus strain, a procedure based on an in situ enzyme-based immunoassay (live black plaque selection) which can detect protein expressed by vaccinia-infected cells was used. Following in vivo recombination on RK-13 cells for 48 hours, RK-13 cells were infected with the viral progeny, and plaques were allowed to form. The cell monolayer was then fixed with 3.7% formaldehyde for 5 minutes, washed once with PBS, and then incubated for 60 minutes with a monoclonal antibody specific for the p17 gag protein (Cellular Products, Inc., Buffalo, NY, cat #0801005) diluted in 3% BSA/PBS. After washing three times in PBS, the sample was incubated for 60 minutes with alkaline phosphate labeled goat anti-mouse IgG (Kirkegard & Perry Laboratories, Gaithersberg, MD) diluted in 3% BSA/PBS, then washed twice with PBS and one with TBS. Color was developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. Positive plaques stain a distinct dark purple color; one plaque was picked and its progeny further propagated. Additional rounds of live black plaque selection were performed on unfixed plaques using IgG purified from serum obtained from HIV-1-infected, human vaccinia naive patients, provided by John Sullivan (University of Massachusetts Medical School, Worcester, MA) as a primary antibody and alkaline phosphatase-labeled, affinity purified goat anti-human IgG as the secondary antibody. The isolated recombinant, designated vAbT4674, was amplified on RK-13 cells and purified over a 36% sucrose cushion.

c. Southern blot analysis of vAbT408 and vAbT4674.

DNA was extracted from vaccinia virus-infected cells as

described (Esposito, et al., J. Virol. Methods 2:175 (1981)) and analyzed by digestion with HindIII and Southern hybridization with radiolabeled probes corresponding to the HIV-1 env or HIV-1 gag-pol genes as described. Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). As expected from predicted genomic structure of these recombinants, hybridization of the digested DNA with radiolabeled gag-pol genes detected fragments of approximately 4992, 1318 and 617 base pairs (bp), while hybridization with radiolabeled env gene detected fragments of approximately 2877 and 1318 bp. This analysis confirmed the presence of these HIV-1 sequences in the recombinant viruses.

EXAMPLE 3

Immunoprecipitation of HIV-1 antigens from cells infected with recombinant vaccinia viruses.

Metabolic labeling with [^{35}S]-methionine of BSC-40 cells infected with recombinant vaccinia viruses vAbT408 and vAbT4674 and subsequent immunoprecipitation analysis was performed essentially as described in U.S. Patent Application Serial No. 910,501, filed September 23, 1986, the teachings of which are incorporated herein by reference. The results, which are summarized in Table 2, show that each of these vaccinia recombinants expresses the encoded polypeptide(s).

TABLE 2

Immunoprecipitation of HIV-1 polypeptides from recombinant vaccinia viruses

Vaccinia recombinants	Inserted genes	Proteins observed
vAbT408, vAbt4674	env, gag-pol	gp160, gp120, gp41 p55, p40, p24, p17 p66, p51, p34

EXAMPLE 4.

Detection, using radioimmunoprecipitation, of Retroviral Particles Produced By Vaccinia Recombinants that express HIV antigens.

Expression analysis described in Example 3 can be used to confirm the synthesis of the polypeptides encoded by inserted HIV genes, but does not address the question of whether these polypeptides self-assemble into retroviral-like particles. As one means of determining whether vaccinia recombinants that express both env and gag-pol produce retroviral-like particles released into the medium of infected cells, the medium was examined for the presence of structures containing env, gag and pol polypeptides which could be pelleted by centrifugation. BSC-40 cells were infected with the recombinant viruses and labeled with [^{35}S]-methionine as described in Example 3. After 16-18 hours of infection, the medium was collected and clarified by centrifugation twice at 3000 rpm for 5 minutes. The resulting supernatant was then centrifuged at 25,000 rpm for 90 minutes. The supernatant was removed, and the resulting pellet was resuspended in 3 ml PBS buffer (136 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4). Samples from the supernatant and pellet were subjected to immunoprecipitation analysis using human anti-HIV antiserum, as described in Example 3. The results showed that while the supernatant contained only gp120, which had been presumably shed into the culture medium during growth of the recombinants (Kieny, et al., Bio/Technology 4:790 (1986)), and the gag-encoded p24 polypeptide, the pellet contained not only gp120, but also the env gene-encoded gp41 as well as the gag gene-encoded p55, p40, p24 and p15, and the pol encoded reverse transcriptase and endonuclease. These results strongly suggested that the recombinant vaccinia-produced env, gag and pol proteins self-assemble into particles or complexes.

EXAMPLE 5.

Analysis of retroviral particles produced by recombinant vaccinia viruses that express HIV antigens using sucrose density gradient sedimentation.

In order to confirm that vaccinia recombinant vAbT408 directs the expression of retroviral-like particles, particles were harvested from the culture medium of 10 roller bottles of BSC-40 cells infected with these recombinants and subjected to sucrose density gradient sedimentation. Cells were infected with the recombinant vaccinia virus at an moi of 10 pfu per cell for 24 hours in serum-free DME. Culture medium was then collected and clarified to remove cell debris by two centrifugations at 3000 rpm for 10 minutes. The cultured medium was filtered through a 0.2μ cellulose acetate filter (Nalgene, cat #156-4020), and particles were then pelleted by centrifugation at 25,000 rpm for 90 minutes in a Beckman SW28 rotor and resuspended in 1 ml STE (10 mM Tris, pH 8, 100 mM NaCl, 1 mM EDTA). 400 μl of the particle preparation was reserved for analysis by SDS-polyacrylamide gel electrophoresis, for measurement of reverse transcriptase activity, and for analysis of RNA content; the remaining 600 μl was applied to a 15 ml 15-45% sucrose density gradient and sedimented for 20 hours at 25,000 rpm in a Beckman SW28 rotor. Fractions (1 ml) were collected and analyzed by SDS-PAGE followed by protein stain or immunoblot using human anti-HIV antiserum. HIV-specific protein bands, including processed gag polypeptides, reverse transcriptase and endonuclease, and envelope glycoproteins, co-sedimented in the gradient; these results demonstrated that the pelleted material contains retroviral-like particles, rather than simply aggregates of retroviral polypeptides. Fractions were also analyzed for reverse transcriptase activity and for the presence of HIV-specific RNA, as described in the following examples.

EXAMPLE 6

Retroviral-like particles produced by HIV/vaccinia recombinants contain HIV-specific RNAs.

The retroviral-like particles described in Example 5, including both the pellet fraction (starting material) as well as the fractions collected from the sucrose density gradient were analyzed for the presence of HIV-specific RNA and for vaccinia-specific RNA. Particle preparations were treated with SDS and proteinase K, then deproteinized by phenol/chloroform extraction. Nucleic acid released by this procedure was concentrated by ethanol precipitation, resuspended in TE buffer, and applied to nitrocellulose filters for hybridization to radiolabeled DNA probes representing the HIV env gene, the HIV gag-pol regions, and the vaccinia TK gene. The results showed that the particles contained nucleic acid which hybridized to both HIV probes used, but not to the vaccinia TK probe.

EXAMPLE 7

Retroviral-like particles produced by recombinant vaccinia viruses contain reverse transcriptase activity.

The retroviral-like particles described in Example 5, including both the pellet fraction (starting material) as well as the fractions collected from the sucrose density gradient were analyzed for the presence of reverse transcriptase (RT) activity. The reaction buffer consisted of 40 mM Tris (pH 7.8), 45 mM KCl, 5 mM $MgCl_2$, 4 m DTT, 0.03% Triton X-100, 25 μg/ml poly rA-oligo dT (Pharmacia cat. #27-7878-02), and 1 uCi ^{3}H-TTP (New England Nuclear, cat. #221H, 1.92 x 10^7 uCi/mMol). Following incubation at 37°C for 60 minutes, the reaction was placed on ice, brought to 25 mM EDTA, precipitated with 6% TCA, and filtered through GFC filters (Whatman). The

filters were washed 2 times with 2% TCA, once with 100% ethanol, dried, and counted in a Beckman LS 1801 scintillation counter. This assay showed that specific RT activity was present in material pelleted from the culture medium of infected cells. Furthermore, this activity co-sedimented with gag and env polypeptides in the sucrose gradient, as expected for HIV-like particles.

EXAMPLE 8

Production of HIV-retroviral-like particles for use as immunogen.

In order to investigate the utility of HIV-like particles produced by recombinant vaccinia virus as an immunogen, particles were prepared from the culture medium of RK-13 cells infected with vAbT408. Twenty-four 15 cm culture dishes of RK-13 cells were infected with vAbT408 at a multiplicity of 10 for 24 hours in serum-free DME. The culture medium was harvested and clarified by two centrifugations at 3000 rpm for 10 minutes, filtered through a 0.45μm filter (Minitan, cat. #HVLPOMP04, Millipore Corp., Bedford, MA) and applied to a discontinuous (20-36-60%) sucrose gradient. The gradient was centrifuged at 25,000 rpm for 5 hours in a Beckman SW28 rotor, and the region containing HIV-like particles was harvested. Particles were concentrated by centrifugation at 25,000 rpm for 12 hours and resuspended in 1.0 ml of PBS. Total protein in the sample was measured using the BioRad Protein Microassay (BioRad cat. #500-0001) according to the manufacturer's instructions. The presence of retroviral-like particles in the preparation was determined by SDS-PAGE analysis followed by staining with Coomassie brilliant blue, which showed the presence of the processed gag polypeptides p24 and p17. The sample was treated with 0.8% formalin overnight to inactivate any residual live vaccinia virus present in the particle preparation.

EXAMPLE 9

Immunopotency of recombinant vaccinia virus vAbT408 and of HIV-like particles derived therefrom.

Recombinant virus vAbT408 was shown to be capable of eliciting HIV-specific humoral immune responses in rabbits. In addition, the HIV-like particle preparation described in Example 8 was shown to be capable of boosting the humoral immune response to HIV in rabbits previously immunized with vaccinia recombinant vAbT408.

Each of the two New Zealand White Rabbits were immunized intravenously with $5x10^7$ pfu of vaccinia recombinant vAbT408. Seventeen weeks after primary immunization with vAbT408, rabbits were boosted with the partially purified particle preparation described in Example 8. Two different adjuvants were used for formulation of the immunogen: complete Freunds adjuvant and alum. Each animal received a total of 5 μg of total particle preparation, administered subcutaneously at five sites on the back. Serum samples were collected at two-week intervals after primary inoculation.

To demonstrate that rabbits inoculated with the recombinant vaccinia virus vAbT408 produced antibodies against authentic HIV-1 proteins, serum samples were analyzed by Western immunoblot. Serum samples diluted 500-fold in Blotto (3% milk, 2% normal goat serum, 0.1% Tween™ 20 in PBS) were reacted with HIV-1 virion proteins which had been resolved by SDS-PAGE and immobilized on nitrocellulose filters by electrotransfer (Epiblot™ HIV strips, Organon Teknika Corp., Durham, NC). HIV-1 proteins recognized by these sera were detected by goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase (Jackson ImmunoResearch). Both animals immunized with the recombinant virus produced antibodies that reacted with HIV-1 p24 gag protein as early as two weeks post-immunization. Weak reactivity to other HIV-1 proteins, including gp160, p66

(reverse transcriptase), and p32 (endonuclease) was also observed. Three weeks following the boost with the particle preparation, both animals developed readily detectable antibody responses to gag (p55, p24 and p17) and pol (p66/51, p32) polypeptides, with the strongest response observed in the animal immunized with particles formulated in complete Freund's adjuvant.

EXAMPLE 10

The immune response in rabbits to particles, alone or in combination with recombinant vaccinia virus (vAbT4674) was investigated. For each immunization with recombinant vaccinia virus, rabbits were vaccinated intradermally with $5x10^7$ pfu. Immunization with particles as described in Example 9. After primary immunization, animals were boosted at weeks 8 and 18. Sera were analyzed by Western blot as described in Example 9. By 24 weeks post-immunization, all animals had developed readily detectable antibody responses to the gag (p55, p24, and p17) and pol (gp160, gp120, and gp41). In order to define more fully the nature of the immune responses elicited by these vaccination protocols, sera were examined for the existence of HIV-1 neutralizing titers by standard serum neutralization assay (Weiss, R.A., et al., Nature 324:572-575 (1986)). The results are set forth below in Table 3.

Table 3

Serum Neutralizing Titers in Rabbits Vaccinated With HIV/Vaccinia Recombinants and HIV-Like Particles

Vaccination Schedule	Rabbit #	Serum Neutralizing Titer					
		Pre	8 wk	10 wk	18 wk	20 wk	24 wk
1° vAbt4674 2° particle/IFA 3° particle/IFA	90-07	<5	5	80	40	160	320
1° vAbt4674 2° vAbT4674 3° particle/IFA	90-13	<5	<5	80	20	80	160
	90-14	<5	5	160	40	80	320
1° particle/IFA 2° particle/IFA 3° particle/IFA	90-15	<5	10	80	10	20	40
	90-16	<5	<5	10	20	160	320
1° vAbT4674 2° particle/alum 3° particle/alum	90-09	<5	5	80	40	40	40
	90-10	<5	<5	10	10	40	20
1° particle/alum 2° particle/alum 3° particle/alum	90-17	5	20	160	80	>640	160
	90-18	20	5	40	20	80	40

EXAMPLE 11

Construction of a recombinant plasmid containing the HIV env gene under the control of the vaccinia 40K promoter for in-vivo recombination with vaccinia virus.

pAbT4716 (Figure 2b)

The construction and structure of pAbT4583 is described in Published International Application No. WO89/12095, published December 14, 1989. The construction and structure pAbT167 is described in Published International Application No. WO90/15141, published December 13, 1990. The teachings of these applications are incorporated herein by reference.

a. Generation of the vector fragment.

pAbT4583 is partially digested with EcoRI and the digestion products electrophoresed on a low melting point agarose gel. A 10719 bp fragment is excised from the agarose gel and purified. The purified linear fragment is next digested with BamHI, and the digestion products electrophoresed on a low melting point agarose gel. A 3586 bp fragment is excised from the gel and purified.

b. Generation of the mutangenized HIV envelope gene fragment.

For mutagenesis, the envelope gene is cloned into a commercially available plasmid, pTZ18U (BioRad, cat. #170-3560). pTZ18U is digested with the restriction enzymes SacI and XbaI, then treated with CIP (calf intestinal phosphatase).

The envelope gene insert is obtained by digesting pAbT167 with SacI and XbaI. The digestion products are electrophoresed on a low melting point agarose gel and the 2700 bp fragment is excised and purified. This fragment is ligated to the pTZ18U

vector, prepared as decribed above, to yield plasmid pAbT8068 (Figure 2a).

Mutagenesis is performed using the BioRad Mutagene Phagemid kit (cat. #170-3576) according to the manufacturer's instructions. The two oligonucleotides used for mutagenesis are as follows:

(5') CAG AAA GAA TAT GCA TTC TTT TAT AAA C (3') and
(5') TGT GGA GGG GAA TTC TTC TAC TGT (3')

The mutagenized plasmid, designated pAbT4705, is shown in Figure 2b. The mutagenesis resulted in the sequence changes indicated in Figure 3, which altered the env gene to remove premature vaccinia transcription termination sequences.

Plasmid pAbT4705 is partially digested with EcoRI and the 5560 bp linear fragment is isolated using a low melting point agarose gel as described above. This isolated linear fragment is next partially digested with BamHI and the resulting 2700 bp BamHI-EcoRI fragment containing the mutagenized envelope gene sequence is gel-purified.

c. Generation of pAbT4716.

The vector fragment described in (a) above, namely the 3586 bp EcoRI-BamHI fragment from pAbT4583, is ligated to the 2700 bp BamHI-EcoRI fragment described in (b) above to generate pAbT4716 (Figure 2b). pAbT4716 a plasmid vector for in vivo recombination with vaccinia virus. pAbT4716 contains the HIV env gene under the transcriptional direction of the vaccinia 40K promoter, which are flanked by sequences from the vaccinia TK gene.

EXAMPLE 12

Construction of a recombinant plasmid containing the HIV gag-pol genes under the control of vaccinia 40K promoter for in-vivo recombination with vaccinia virus.

pAbT4704 (Figure 4b)

The construction and structure and pAbT598 is described in International Application No. WO90/15141, published December 13, 1990. The teaching of this application is incorporated herein by reference.

The IVR vector pAbT4555 was decribed in International Application No. WO89/12095, published December 14, 1989, the teaching of which is incorporated herein by reference. A derivative of this vector was constructed in the following manner. pAbT4555 was digested with HincII and BgIII, and a 110 bp fragment resulting from this digestion was gel-purified. In a second reaction, pAbT4555 was digested with SphI, treated with T4 DNA polymerase, and then digested with BgIII. A 3856 bp fragment resulting from this digestion was gel-purified. The 110 bp fragment and the 3856 bp fragment were ligated to yield the plasmid pAbT4587 (Figure 4a).

pAbT4587 is identical to pAbT4555, except that DNA sequences containing the 30K promoter that are present in pAbT4555 were deleted.

a. Construction of pAbT623.

Plasmid pAbT598 was digested with BamHI and SacI, and a 4340 bp fragment resulting from this digestion was gel-purified. In a separate reaction, pAbT4587 was digested with BamHI and SacI, and treated with CIP. This vector was ligated to the 4340 bp fragment from pAbT598 to yield pAbT623 (Figure 4a and 4b).

b. Construction of pAbT4703.

pAbT623 was digested with PstI and a 798 bp fragment resulting from the digestion was gel-purified. In a separate reaction, plasmid pAbT4672, described above in Example 1a (Figure 1a), was digested with PstI and treated with CIP. This vector was ligated to the 798 bp fragment from pAbT623 to yield pAbT4703 (Figure 4b).

c. Construction of pAbT4704.

pAbT4703 was digested with SacI and ligated to the following linkers

5'TTTTTATAAAAAGAGCTCCCCGGGTAGCT3'
3'TCGAAAAAATATTTTTCTCGAGGGGCCCA5'

The resulting plasmid was designated pAbT4704 (Figure 4b). pAbT4704 is a plasmid vector for in-vivo recombination with vaccinia virus. pAbT4704 contains the HIV gag-pol coding sequence (but not the cis-acting packaging sequences 5' to the gag initiation codon) under the transcriptional direction of the vaccinia 40K promoter, which are flanked from the HindIII M region of vaccinia virus.

EXAMPLE 14

Construction of a recombinant vaccinia virus containing the HIV gag-pol and env genes.

In vivo recombination (IVR) was performed as described above in Example 2a.

IVR vector pAbT4704 was transfected into BSC-40 cells which had been infected with vaccinia virus vAbT33. Viral infection and plasmid transfection were performed as described above. Recombinant viruses were selected as white plaques in the presence of Bluogal on RK-13 cells. Plaques were picked and purified, and the final recombinant, desginated vAbT480, was amplified on RK-13 cells and purified over a 36% sucrose cushion.

Plasmid pAbT4716 was inserted into the vaccinia virus genome at the TK locus of vAbT480 using a selection scheme based on the sensitivity of the TK^+ viruses to bromodeoxyuridine (BUdR), as described in International Publication WO90/15141. The resulting recombinant vaccinia virus was designated vAbT489.

Plasmid Deposits

The plasmid vAbT4674 was placed on deposit, under provisions of the Budapest Treaty, at the American Type Culture Collection in Rockville, Maryland on June 14, 1990. The plasmid has been assigned accession number 40829.

The plasmids pAbT4704 and pAbT4716 were placed on deposit, under the provision of the Budapest Treaty, at the American Type Culture Collection in Rockville, Maryland on June 19, 1991, and were assigned accession number _____ and _____, respectively.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A recombinant DNA viral vector corresponding to a sufficient portion of a pox virus genome to coexpress heterologous viral polypeptides, wherein said vector coexpresses, in eukaryotic cells, env and gag-pol retroviral genes or modified versions thereof, encoding retroviral envelope, gag and pol polypeptides capable of self-assembly into defective nonself-propagating retroviral particles.

2. The recombinant viral vector of Claim 1, wherein the pox virus is a vaccinia virus.

3. A recombinant viral vector of Claim 1, wherein the retroviral genes are lentiviral genes.

4. A recombinant viral vector of Claim 3, wherein the lentivirus is a human immunodeficiency virus.

5. A recombinant viral vector of Claim 1, wherein the coexpressed retroviral polypeptides assemble into particles having a substantially reduced RNA content.

6. A recombinant viral vector of Claim 1, wherein the coexpressed retroviral polypeptides assemble into particles having modified transcribable RNA incorporated therein.

7. A recombinant viral vector of Claim 6, wherein the modified RNA encodes a heterologous gene product.

8. A recombinant viral vector of Claim 1, wherein the coexpressed retroviral polypeptides assemble into particles having modified nontranscribable RNA incorporated therein.

9. A recombinant vector which comprises a sufficient portion of a vaccinia virus genome to coexpress, in eukaryotic cells, a DNA segment corresponding to an HIV env gene, wherein the env gene has been altered to remove premature vaccinia transcription termination sequences, and a DNA segment corresponding to a HIV gag-pol gene, wherein the 5' packaging sequence has been deleted.

10. The vector of Claim 9, wherein the products of the coexpressed genes, HIV envelope, gag and pol polypeptides, are capable of self-assembly into defective nonself-propagating viral particles.

11. A self-assembled defective nonself-propagating retroviral particle expressed by a eukaryotic cell infected by a recombinant DNA viral vector which coexpresses the env and gag-pol retroviral genes or modified versions thereof, encoding retroviral envelope, gag and pol polypeptides capable of self-assembly into the particle.

12. A retroviral particle of Claim 11, wherein the DNA virus is a pox virus.

13. A retroviral particle of Claim 12, wherein the pox virus is a vaccinia virus.

14. A retroviral particle of Claim 11, wherein the retroviral genes are lentiviral genes.

15. A retroviral particle of Claim 14, wherein the retroviral genes are human immunodeficiency viral genes.

16. A retroviral particle of claim 11, wherein the retroviral particle elicits a cell mediated immune response.

17. A self-assembled defective nonself-propagating retroviral particle having a substantially reduced RNA content or modified expressible RNA incorporated therein, expressed by a eukaryotic cell infected by a recombinant DNA viral vector which coexpresses the env and gag-pol retroviral genes or modified versions thereof encoding retroviral envelope, gag and pol polypeptides capable of self-assembly into the particle.

18. A retroviral particle of Claim 17, wherein the retroviral genes are human immunodeficiency viral genes.

19. A retroviral particle of Claim 17, wherein the modified RNA encodes a heterlogous gene product.

20. A retroviral particle of Claim 19, wherein the heterologous gene product is a therapeutic agent.

21. A retroviral particle of Claim 20, wherein the therapeutic agent is an antiviral agent or cytotoxic agent.

22. A retroviral particle of Claim 17, wherein the modified RNA is transcribable RNA.

23. A retroviral particle of Claim 17, wherein the modified RNA is nontranscribable RNA.

24. A vaccine composition comprising an immunizing amount of a recombinant DNA viral vector of Claim 1 in a pharmaceutically acceptable vehicle.

25. A vaccine composition of Claim 24, wherein the DNA viral vector is a recombinant vaccinia virus which coexpresses the env and gag-pol genes of human immunodeficiency virus.

26. A vaccine composition of Claim 24, further comprising self-assembled defective nonself-propagating particles having retroviral envelope, gag and pol polypeptides.

27. A vaccine composition comprising an immunizing amount of self-assembled defective nonself-propagating retroviral particles of Claim 11, in a pharmaceutically acceptable vehicle.

28. A vaccine composition of Claim 27, wherein the retroviral genes are human immunodeficiency viral genes.

29. A vaccine composition comprising an immunizing amount of self-assembled defective nonself-propagating retroviral particles of Claim 17, in a pharmaceutically acceptable vehicle.

30. A method of immunizing against a retrovirus, comprising inoculating a host to be immunized with a recombinant DNA viral vector which coexpresses env and gag-pol retroviral genes or modified versions thereof, encoding envelope, gag and pol polypeptides capable of self-assembly into a defective nonself-propagating retroviral particle, in a pharmaceutically acceptable vehicle.

31. A method of Claim 30, wherein the recombinant DNA viral vector is a vaccinia virus and the retroviral genes are human immunodeficiency genes.

32. A method of Claim 30, further comprising, administering to the host an immunizing amount of a self-assembled defective nonself-propagating retroviral particle having retroviral envelope, gag and pol polypeptides, in a pharmaceutically acceptable vehicle.

33. A method of Claim 32, wherein the retroviral particle comprises human immunodeficiency viral envelope, gag and pol polypeptides.

34. A method of Claim 32, wherein the retroviral particle has a substantially reduced RNA content.

35. A targeted therapeutic agent comprising a therapeutic agent linked to, or incorporated within, a viral particle of Claim 17.

36. A targeted therapeutic agent of Claim 35, wherein the therapeutic agent is RNA encoding an antiviral agent or a cytotoxic agent.

37. A targeted therapeutic agent of Claim 35, wherein the viral particle comprises HIV envelope, gag and pol polypeptides.

38. A method of targeting the delivery of a therapeutic agent to a virally infected cell, comprising administering with therapeutic agent linked to, or incorporated within, a viral particle of Claim 17, in a pharmaceutically acceptable vehicle.

39. A method of Claim 38, wherein the virally infected cell is an HIV-infected, CD4 receptor-bearing cell and the viral particle comprises HIV envelope, gag and pol polypeptides.

40. A method of Claim 39, wherein the therapeutic agent is HIV RNA encoding an antiviral or cytotoxic agent.

41. A method of Claim 38, wherein the therapeutic agent is RNA encoding a heterlogous gene product.

42. Recombinant vaccinia virus vAbT408, vAbT4674 or vAbT489.

43. Plasmid DNA vector pAbT4674 having an ATCC designation number 40829.

44. Plasmid DNA vector pAbT4704 having an ATCC designation number _____.

45. Plasmid DNA vector pAbT4716 having an ATCC designation number _____.

46. A method of substantially purifying a self-assembled defective nonself-propagating viral particle expressed by a eukaryotic cell infected by a recombinant DNA viral vector which coexpresses at least two genes encoding heterologous viral polypeptides capable of self-assembly into the particle, comprising the steps of:

a) subjecting a sample comprising the viral particle to be purified to a sucrose density gradient;

b) separating the particle from sample according to density by sedimentation in the sucrose gradient; and

c) collecting the sedimented particle which is thereby substantially purified.

47. A purification method of Claim 46, wherein the genes encoding viral polypeptides are retroviral genes.

48. A purification method of Claim 47, wherein the retroviral genes are human immunodeficiency viral genes.

49. A purification method of Claim 46, wherein the recombinant viral vector is a vaccinia virus and the genes encoding heterologous polypeptides are retroviral env and gag-pol genes.

50. A DNA vector for insertion of retroviral cDNA into a DNA viral vector by in vivo recombination comprising:
    a) a prokaryotic origin of replication so that the vector may be amplified in the prokaryotic host;
    b) a gene encoding a marker which allows selection of prokaryotic host cells that contain the vector;
    c) env and gag-pol DNA sequences encoding retroviral envelope, gag and pol polypeptides capable of self-assembly into defective nonself-propagating retroviral particles, each DNA sequence located adjacent to a transcriptional promoter; and
    d) DNA sequence homologous to the region of the DNA viral genome where the retroviral cDNA sequences will be inserted, flanking the construct of element c.

51. A DNA vector of Claim 50, wherein the retroviral DNA is human immunodeficiency viral DNA.

52. A DNA vector of Claim 50, wherein the gag gene encodes polypeptides deficient in an attachment site for genomic RNA.

53. A DNA vector of Claim 50, wherein the gag-pol DNA sequence has a cis-acting packaging sequence deleted therefrom.

54. A DNA vector for insertion of retroviral cDNA into a recombinant DNA viral vector by in vivo recombination, wherein the recombination viral vector is capable of coexpressing in eukaryotic cells, env and gag-pol retroviral genes, encoding envelope, gag and pol polypeptides capable of self-assembly into defective nonself-propagating retroviral particles having RNA encoding a heterologous gene product incorporated therein, the DNA vector comprising:
    a) a prokaryotic origin of replication so that the vector may be amplified in the prokaryotic host;

b) a gene encoding a marker which allows selection of prokaryotic host cells that contain the vector;

c) env and gag-pol DNA sequences encoding retroviral envelope, gag and pol polypeptides capable of self-assembly into defective nonself-propagating retroviral particles, each DNA sequence located adjacent to a transcriptional promoter;

d) in 5' and 3' order a eukaryotic promoter, a retroviral long terminal repeat, a cis-acting packaging sequence, a eukaryotic promoter, a DNA sequence encoding a heterologous gene product, a retroviral long terminal repeat and an RNA termination sequence; and

e) DNA sequence homologous to the region of the DNA viral genome where the retroviral cDNA sequences will be inserted, flanking the construct of elements c and d.

55. A DNA vector of Claim 54, wherein the retroviral DNA is human immunodeficiency viral DNA.

56. A DNA vector of Claim 55, wherein the heterlogous gene product is a therapeutic agent.

57. A DNA vector for insertion of retroviral cDNA into a recombinant DNA viral vector by in vivo recombination, wherein the recombinant viral vector is capable of coexpressing in eukaryotic cells, env and gag-pol retroviral genes, encoding envelope, gag and pol polypeptides capable of self-assembly into defective nonself-propagating retroviral particles having nontranscribable RNA encoding a heterologous gene product incorporated therein, the DNA vector comprising:

a) a prokaryotic origin of replication so that the vector may be amplified in the prokaryotic host;

b) a gene encoding a marker which allows selection of prokaryotic host cells that contain the vector;

c) env and gag-pol DNA sequences encoding retroviral envelope, gag and pol polypeptides capable of self-assembly into defective nonself-propagating retroviral particles, each DNA sequence located adjacent to a transcriptional promoter;

d) a cis-acting packaging sequence linked to a DNA sequence encoding a heterologous gene product; and

e) DNA sequence homologous to the region of the DNA iral genomie where the retroviral cDNA sequences will be inserted, flanking the construct of elements c and d.

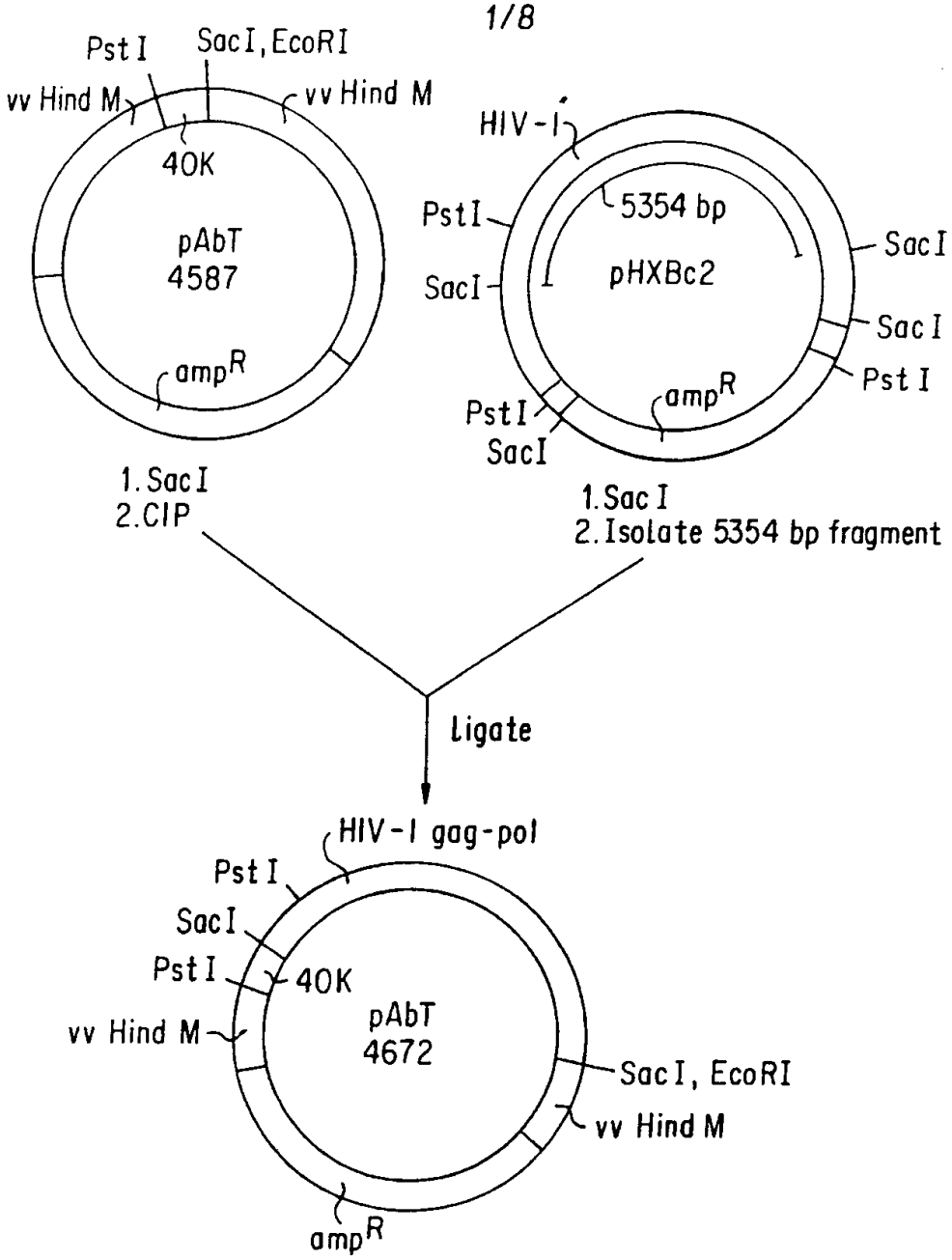
Pst I
Sac I, EcoRI
vv Hind M
vv Hind M
40K
pAbT
4587
amp^R
1. Sac I
2. CIP
HIV-1
5354 bp
pHXBc2
Pst I
Sac I
Sac I
Sac I
Pst I
Pst I
Sac I
amp^R
1. Sac I
2. Isolate 5354 bp fragment
Ligate
HIV-1 gag-pol
Pst I
Sac I
Pst I
40K
vv Hind M
pAbT
4672
Sac I, EcoRI
vv Hind M
amp^R

FIG. 1a

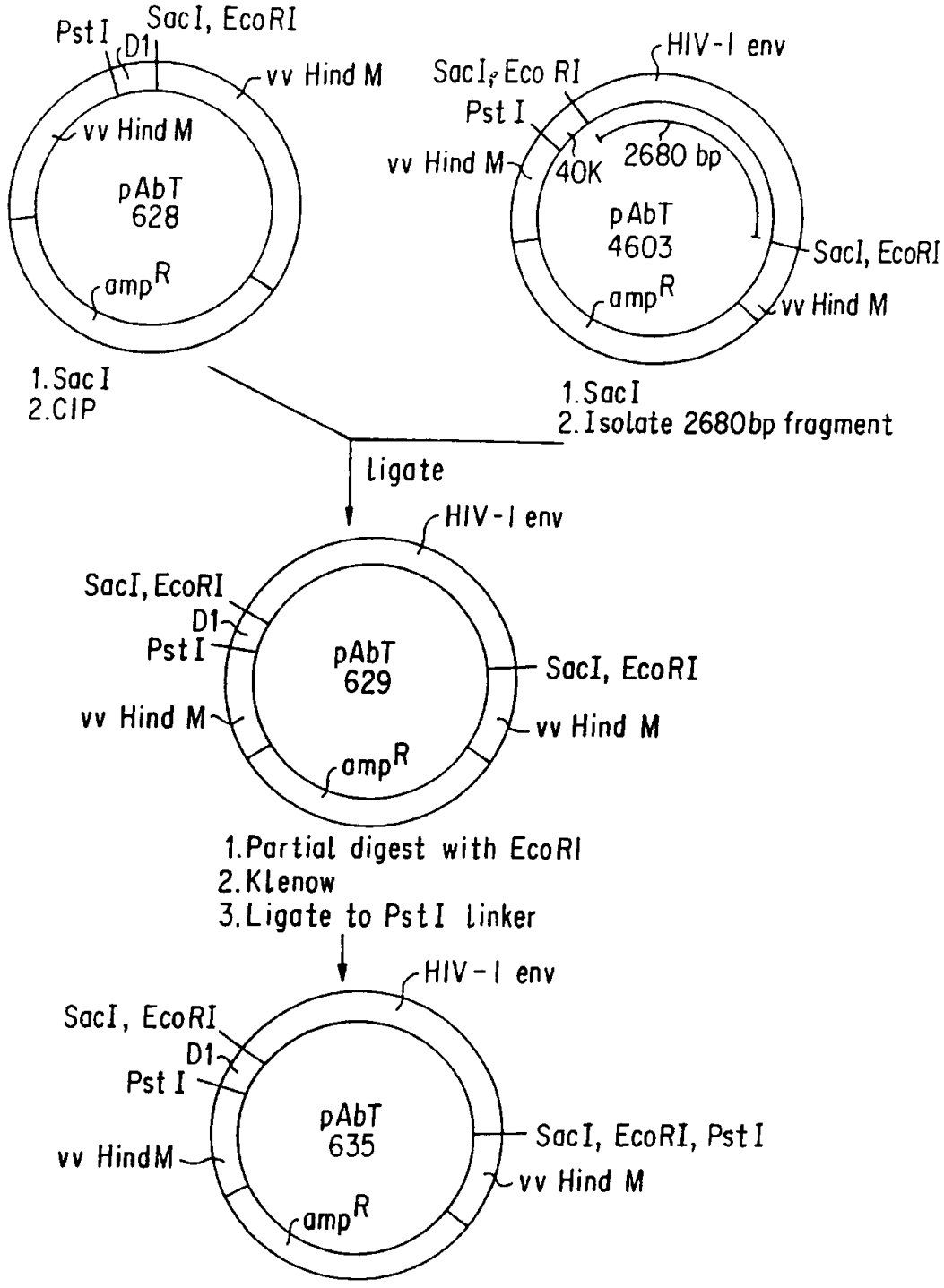
Pst I
D1
Sac I, Eco RI
vv Hind M
vv Hind M
pAbT
628
amp^R
1. Sac I
2. CIP
Sac I, Eco RI
HIV-1 env
Pst I
vv Hind M
40K
2680 bp
pAbT
4603
amp^R
Sac I, Eco RI
vv Hind M
1. Sac I
2. Isolate 2680bp fragment
ligate
HIV-1 env
Sac I, Eco RI
D1
Pst I
vv Hind M
pAbT
629
amp^R
Sac I, Eco RI
vv Hind M
1. Partial digest with Eco RI
2. Klenow
3. Ligate to Pst I linker
HIV-1 env
Sac I, Eco RI
D1
Pst I
vv Hind M
pAbT
635
amp^R
Sac I, Eco RI, Pst I
vv Hind M

FIG. 1b

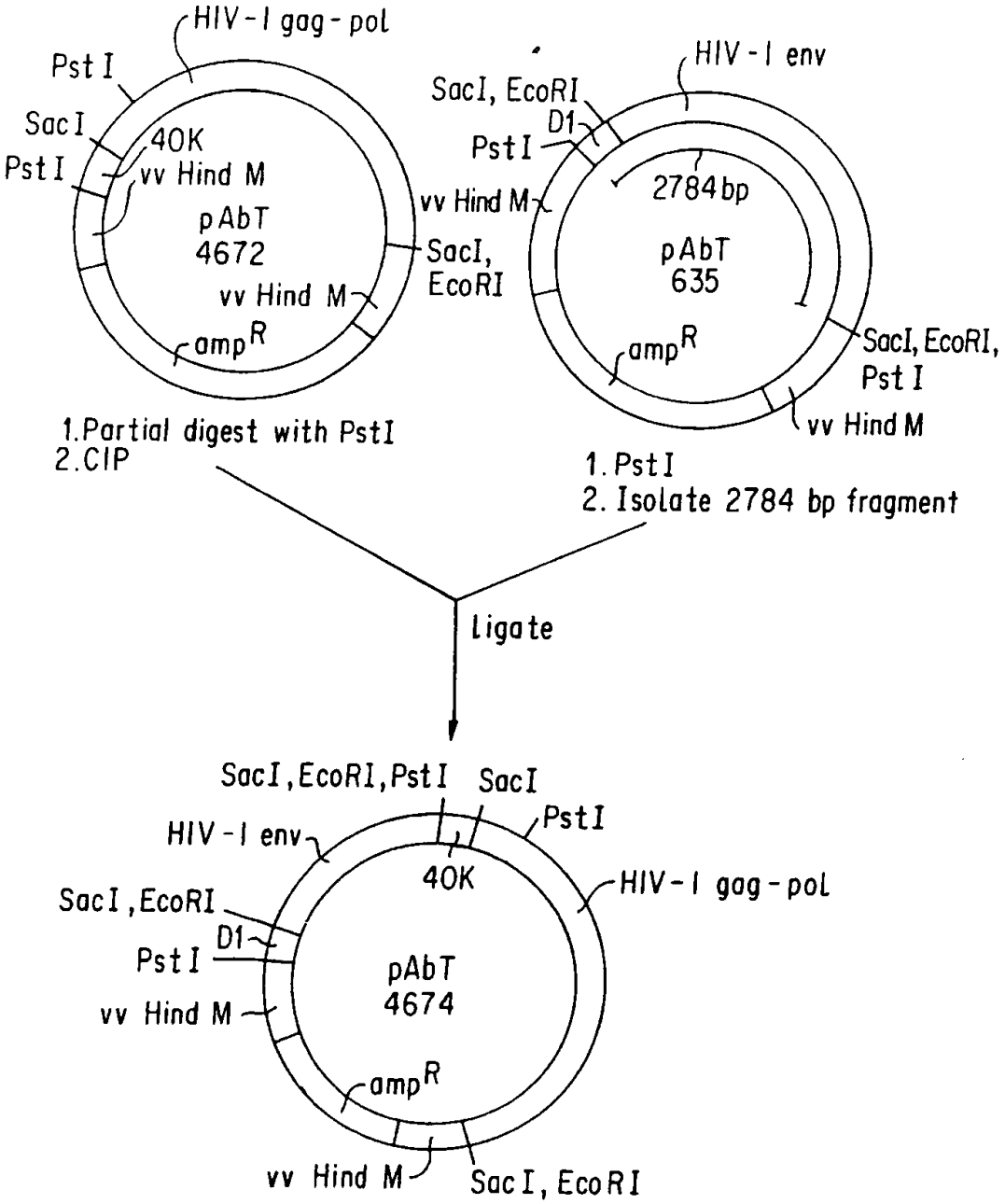
HIV-1 gag-pol
Pst I
Sac I
40K
Pst I
vv Hind M
pAbT
4672
Sac I,
EcoRI
vv Hind M
amp R
1. Partial digest with PstI
2. CIP
HIV-1 env
Sac I, EcoRI
D1
Pst I
vv Hind M
2784 bp
pAbT
635
amp R
Sac I, EcoRI,
Pst I
vv Hind M
1. Pst I
2. Isolate 2784 bp fragment
Ligate
Sac I, EcoRI, Pst I
Sac I
Pst I
HIV-1 env
40K
HIV-1 gag-pol
Sac I, EcoRI
D1
Pst I
vv Hind M
pAbT
4674
amp R
vv Hind M
Sac I, EcoRI

FIG. 1c

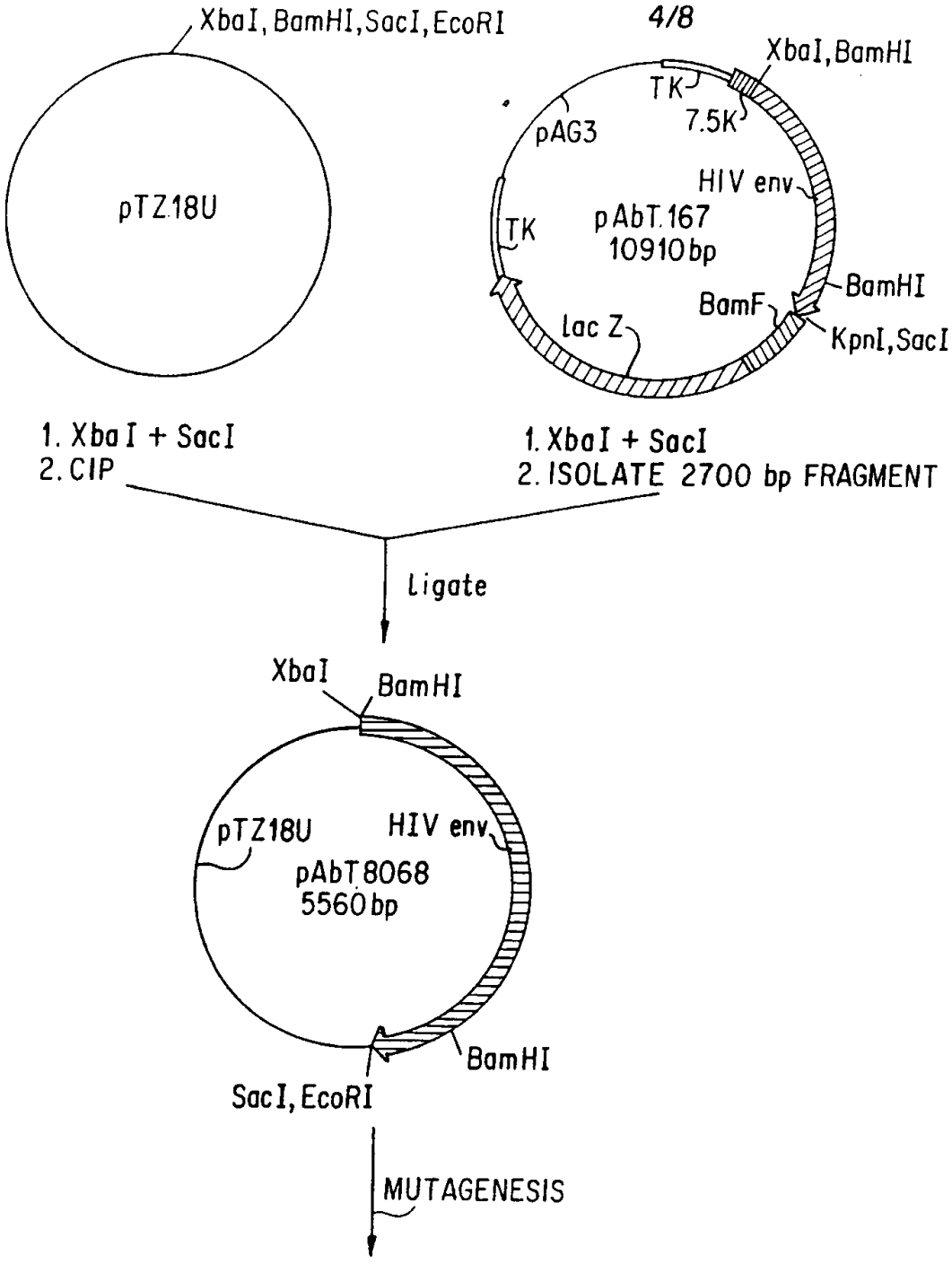
XbaI, BamHI, SacI, EcoRI
pTZ18U
1. XbaI + SacI
2. CIP
XbaI, BamHI
TK
7.5K
pAG3
HIV env
TK
pAbT167
10910bp
BamHI
BamF
lac Z
KpnI, SacI
1. XbaI + SacI
2. ISOLATE 2700 bp FRAGMENT
Ligate
XbaI
BamHI
pTZ18U
HIV env
pAbT8068
5560bp
BamHI
SacI, EcoRI
MUTAGENESIS

FIG. 2a

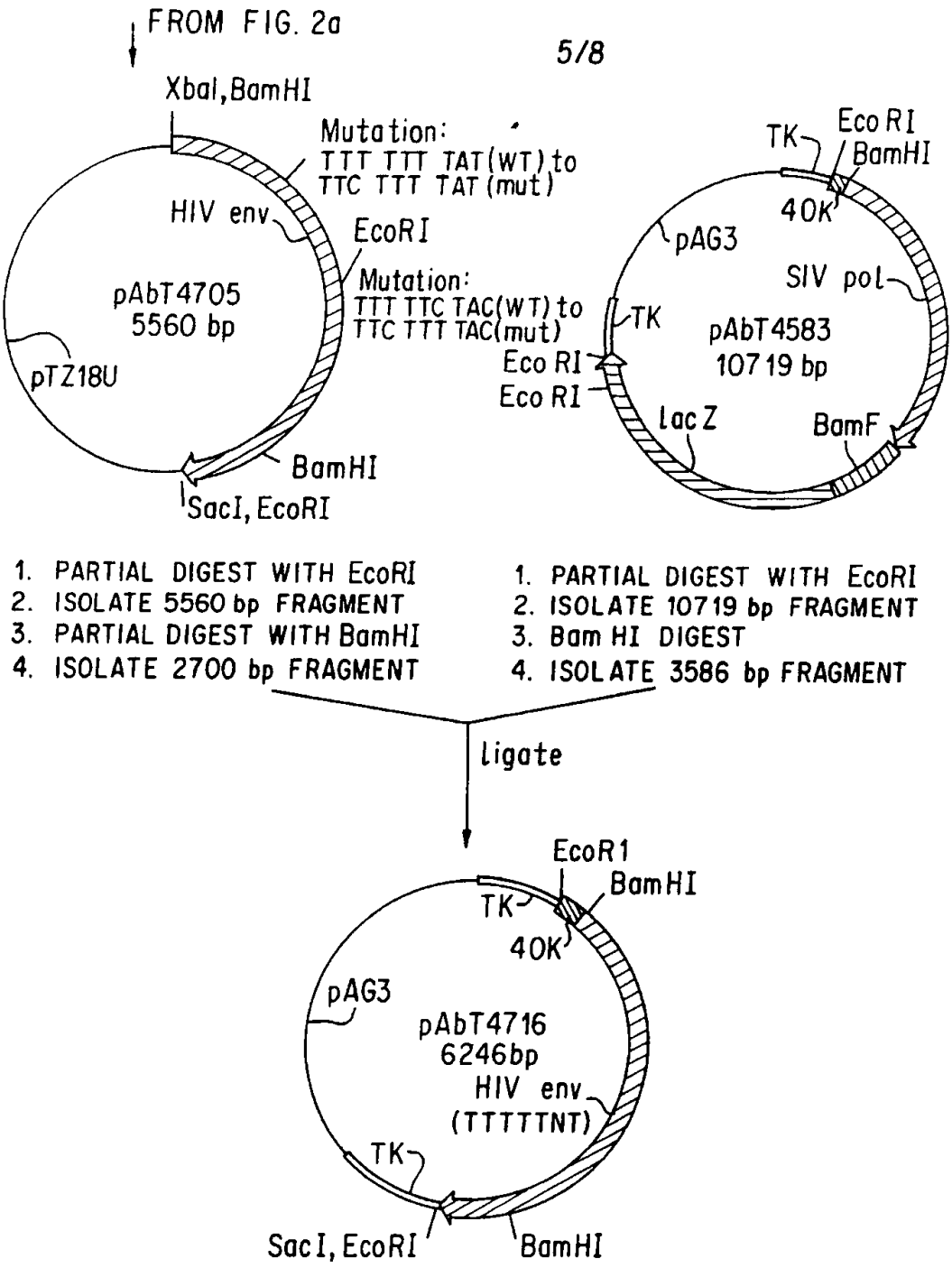
FROM FIG. 2a
XbaI, BamHI
Mutation:
TTT TTT TAT(WT) to
TTC TTT TAT (mut)
HIV env
EcoRI
pAbT4705
5560 bp
pTZ18U
Mutation:
TTT TTC TAC(WT) to
TTC TTT TAC(mut)
BamHI
SacI, EcoRI
TK
Eco RI
BamHI
40K
pAG3
SIV pol
TK
pAbT4583
10719 bp
Eco RI
Eco RI
lac Z
BamF
1. PARTIAL DIGEST WITH EcoRI
2. ISOLATE 5560 bp FRAGMENT
3. PARTIAL DIGEST WITH BamHI
4. ISOLATE 2700 bp FRAGMENT
1. PARTIAL DIGEST WITH EcoRI
2. ISOLATE 10719 bp FRAGMENT
3. Bam HI DIGEST
4. ISOLATE 3586 bp FRAGMENT
ligate
EcoR1
BamHI
TK
40K
pAG3
pAbT4716
6246bp
HIV env
(TTTTTNT)
TK
SacI, EcoRI
BamHI

FIG. 2b

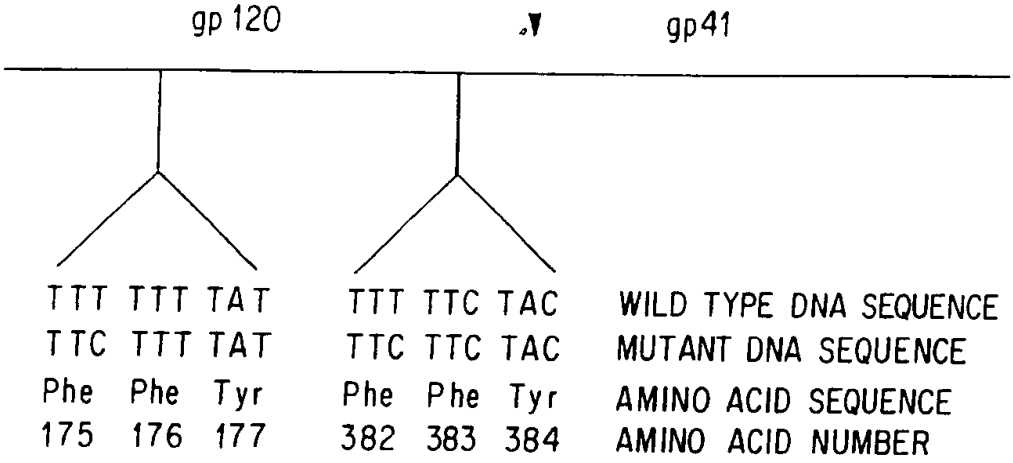
gp 120
gp41
TTT TTT TAT
TTC TTT TAT
Phe Phe Tyr
175 176 177
TTT TTC TAC
TTC TTC TAC
Phe Phe Tyr
382 383 384
WILD TYPE DNA SEQUENCE
MUTANT DNA SEQUENCE
AMINO ACID SEQUENCE
AMINO ACID NUMBER

FIG. 3

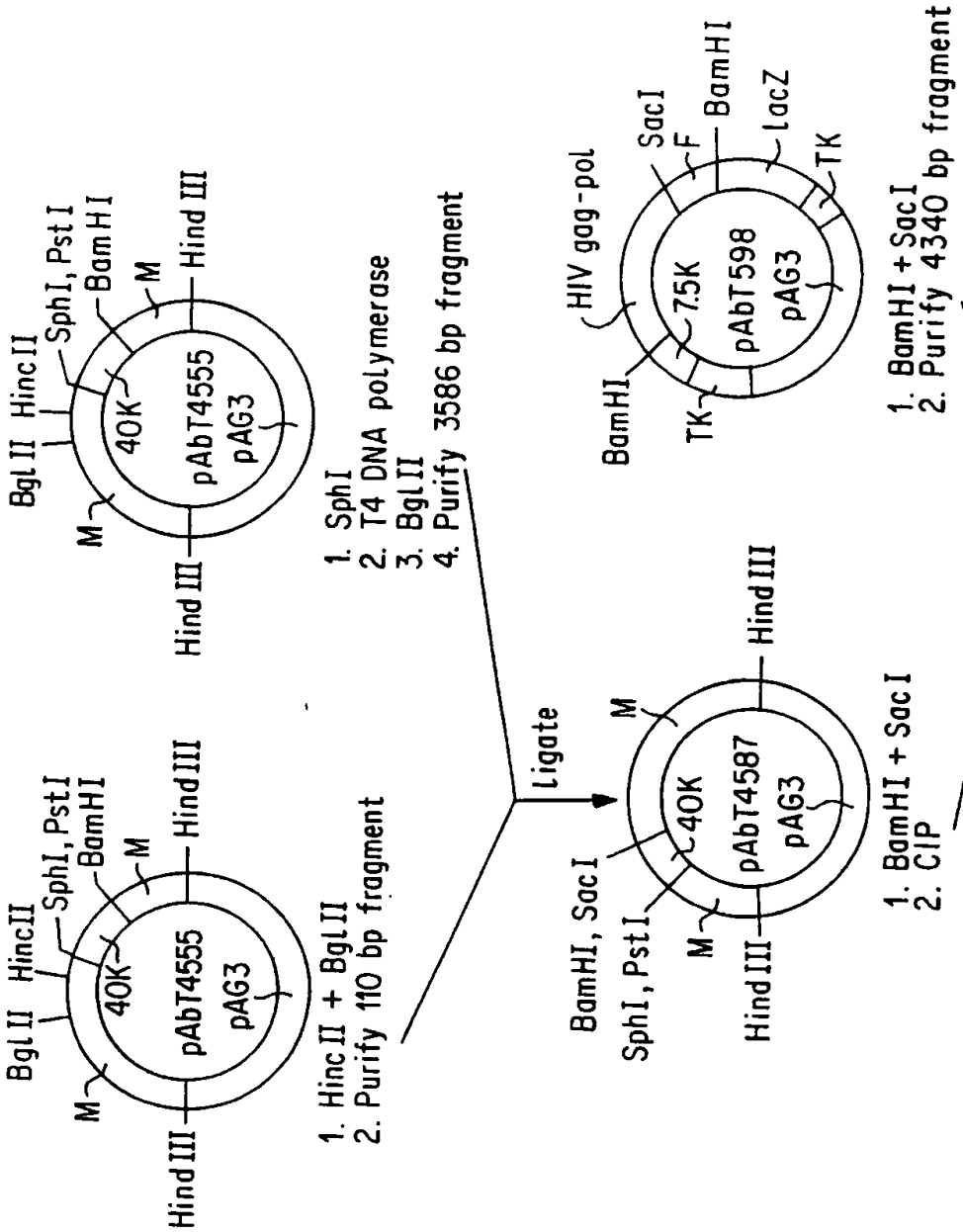
Bgl II
HincII
SphI, PstI
BamHI
M
Hind III
40K
pAbT4555
pAG3
1. HincII + BglII
2. Purify 110 bp fragment
1. SphI
2. T4 DNA polymerase
3. BglII
4. Purify 3586 bp fragment
Ligate
BamHI, SacI
SphI, PstI
M
Hind III
40K
pAbT4587
pAG3
1. BamHI + SacI
2. CIP
Ligate
HIV gag-pol
SacI
F
BamHI
lacZ
TK
7.5K
pAbT598
pAG3
1. BamHI + SacI
2. Purify 4340 bp fragment

FIG. 4a

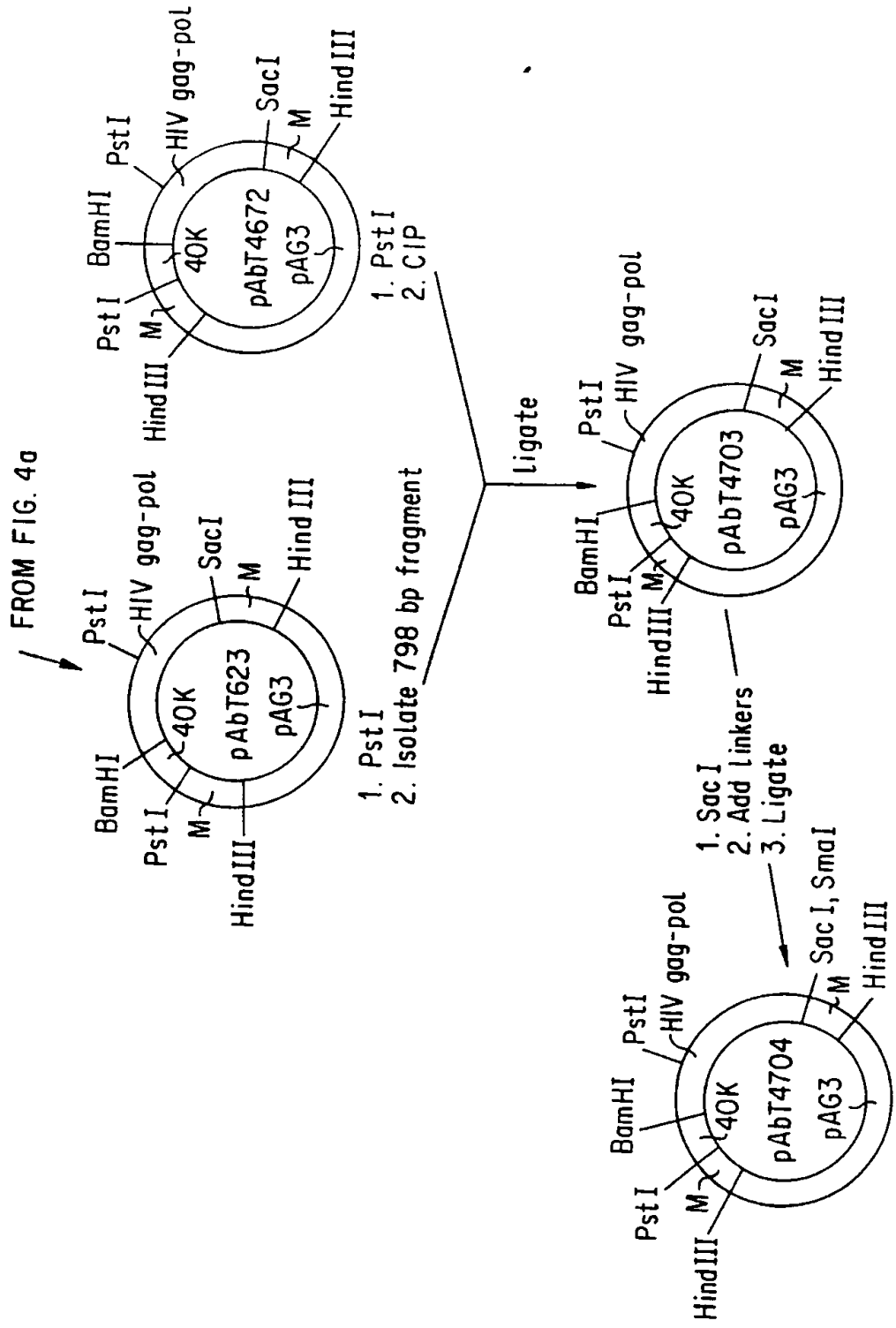
FROM FIG. 4a
Pst I
HIV gag-pol
Sac I
M
Hind III
BamHI
Pst I
M
Hind III
40K
pAbT623
pAG3
1. Pst I
2. Isolate 798 bp fragment
Pst I
HIV gag-pol
Sac I
M
Hind III
BamHI
Pst I
M
Hind III
40K
pAbT4672
pAG3
1. Pst I
2. CIP
Ligate
Pst I
HIV gag-pol
Sac I
M
Hind III
BamHI
Pst I
M
Hind III
40K
pAbT4703
pAG3
1. Sac I
2. Add linkers
3. Ligate
Pst I
HIV gag-pol
Sac I, Sma I
M
Hind III
BamHI
Pst I
M
Hind III
40K
pAbT4704
pAG3

FIG. 4b

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/04372

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 N 15/49 C 12 N 15/86 A 61 K 39/21
A 61 K 47/00 C 07 K 13/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 12 N C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	WO,A,9015141 (APPLIED BIOTECHNOLOGY INC.) 13 December 1990, see the whole document; claims 5-11,15-18,21-47 (cited in the application)	1-4,7, 11-15, 24-28, 30-33, 35-38, 50-51
P,Y		30-33

P,X	WO,A,9107425 (ONCOGEN LIMITED PARTNERSHIP) 30 May 1991, see the whole document	1-4,11-15,24-28
P,X		30-33, 35-38

P,Y	WO,A,9106658 (CETUS CORPORATION) 16 May 1991, see the whole document	35-38, 11-18
	--- -/-	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15-10-1991	11. 11. 91
International Searching Authority: EUROPEAN PATENT OFFICE	Signature of Authorized Officer: D. Frank Mme Dagmar FRANK

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

	Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
5	P,X	WO,A,9105864 (CONAUGHT LABORATORIES LTD) 02 May 1991, see the whole document	11-16, 27-28, 46-48
5	P,X	WO,A,9105860 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 02 May 1991, see the whole document	11-18, 29
	P,X		35-38
5	X	EP,A,0381146 (BANYU PHARMACEUTICAL CO.) 08 August 1991, see claim 6,8-9; example 1	11-18, 29
4	Y	EP,A,0243204 (CETUS CORPORATION) 28 October 1987, see the whole document	35-38
5	Y	WO,A,8803563 (OXFORD GENE SYSTEMS LIMITED) 19 May 1988, see the claims	11-18, 35-38

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9104372 SA 49867

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 04/11/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 9015141	13-12-90	AU-A- 5848390	07-01-91
		EP-A- 0431128	12-06-91
WO-A- 9107425	30-05-91	AU-A- 6905591	13-06-91
WO-A- 9106658	16-05-91	AU-A- 6956691	31-05-91
WO-A- 9105864	02-05-91	None	
WO-A- 9105860	02-05-91	None	
EP-A- 0381146	08-08-90	JP-A- 2203783	13-08-90
EP-A- 0243204	28-10-87	JP-A- 63157985	30-06-88
WO-A- 8803563	19-05-88	AU-B- 606896	21-02-91
		AU-A- 8105787	01-06-88
		AU-B- 606627	14-02-91
		AU-A- 8153487	01-06-88
		EP-A- 0330661	06-09-89
		EP-A- 0329671	30-08-89
		WO-A- 8803562	19-05-88
		JP-T- 2501107	19-04-90
		JP-T- 2501026	12-04-90
		US-A- 5041385	20-08-91
		US-A- 5008373	16-04-91